Apoptosis induced by modified ribonucleosides in human cell culture systems

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Abstract The in vitro modulation of apoptosis and cell proliferation by modified in comparison with non-modified ribonucleosides was investigated for the first time using peripheral blood lymphocytes, HL-60 cells and Caco-2 cells as human cell culture models. Modulating effects of several ribonucleosides were found in the range of 10^{-7}–10^{-3} mol/l. The following ribonucleosides induced significant apoptosis of HL-60 cells: adenosine, N^6-dimethyladenosine, N^6-(2-isopentenyl)-adenosine, N^2-dimethylguanosine. A significant apoptotic effect on PBL was found with N^6-dimethyladenosine and N^6-(2-isopentenyl)-adenosine. N^6-Dimethyladenosine, N^6-(2-isopentenyl)-adenosine and guanosine had a pronounced inhibitory effect on Caco-2 cell apoptosis. Regarding the known function of ribonucleosides as pathobiochemical marker molecules for cancer, the possibility of a selective apoptotic effect against malignant cells is discussed.

Key words: Ribonucleoside; Human cell culture; Apoptosis; Proliferation; Anticancerogen

1. Introduction

Besides their central role as monomeric components of nucleic acids, nucleos(t)ides and nucleobases have several regulatory functions (for review: [1,2]), e.g. they affect immune modulation, contribute to iron absorption in the gut and influence desaturation and elongation rates in fatty acid synthesis. Apart from these bio- and trophochemical properties, ribonucleosides can influence the activity of cells due to their translocation across the plasma membrane [3] by specified facilitated-diffusion carriers and sodium-dependent translocators [3–6] located in intestinal and kidney epithelial cells [7,8]. Modified ribonucleosides excreted in higher amounts in the urine of cancer patients could serve as pathobiochemical marker molecules for different types of cancer diseases [9–12].

Although there is growing interest in the role of apoptosis in cancer, the possible involvement of modified ribonucleosides as signals that can trigger apoptosis of cancer cells has not yet been investigated. Apoptosis is a type of cell death exhibiting a distinct set of morphological and biochemical changes (for review: [13]). Deficient apoptosis can promote tumorigenesis, both by allowing the accumulation of dividing cells and by not removing cells with genetic lesions [14]. In this study, the modulation of apoptosis and cell proliferation by different ribonucleosides is described in human cell culture models. Differences are apparent for the apoptotic effect of modified compared to non-modified ribonucleosides.

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2. Materials and methods

2.1. Ribonucleosides

N^6-Carbamoyl-threonyladenosine (t6ado) was synthesized according to [15]; N^2-dimethylguanosine (m2,2guo) was a gift from K.-P. Raezke (Applica GmbH, Bremen, Germany). All other ribonucleosides such as adenosine (ado), N^1-methyladenosine (m1ado), N^6-methyladenosine (m6ado), N^6-dimethyladenosine (m6,2ado), N^6-(2-isopentenyl)-adenosine (ip6ado), N^2-dimethylguanosine (m2,2guo), N^7-methylguanosine (m7guo), inosine (ino), N^1-methylinosine (m1ino), N^7-methylinosine (m7ino), cytidine (cyd) and uridine (urd) were obtained from Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany. The ribonucleoside content in different preparations as well as in culture media was analyzed by HPLC as described in [16] using a photodiode array detector. Ribonucleoside preparations with a purity of >99% were used for cell chemical analyses.

2.2. Cells and culture conditions

Human peripheral blood lymphocytes (PBL) were isolated from heparin-treated blood of healthy human volunteers by density gradient centrifugation method as described [17]. HL-60 cells (promyelocytic leukemia, human) were from the American Type Culture Collection, Rockville, MD, USA (ATCC CCL-240) and Caco-2 cells from DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany (DSM ACC 169). Cells were routinely grown in 25 cm^2 plastic flasks (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) at 37°C in an atmosphere of 5% CO_2. PBL and HL-60 cells were maintained in suspension culture in a low protein medium HL-1 (BioWhittaker Inc., Walkersville, USA) supplemented with 0.5% heat-inactivated fetal calf serum (FCS; Sigma-Aldrich) and 2 mmol/l L-glutamine. Caco-2 cells were cultured in Dulbecco's modified Eagle medium (DMEM; Sigma-Aldrich) with 4.5 g/l glucose supplemented with 20% FCS and 4 mmol/l L-glutamine. All culture media contained 100 U/ml penicillin and 100 μg/ml of streptomycin (Sigma-Aldrich). HL-60 cells were seeded at 1×10^5 cells/ml and were passaged every 3–4 days.

Caco-2 cells plated at 1×10^4 cells/cm^2 were passaged every 4–5 days when confluence was reached; medium was changed every 2 days. The Caco-2 monolayer was dissociated using 0.05% trypsin-0.02% EDTA solution in Hanks' balanced salt solution (HBSS; Sigma-Aldrich). HL-60 and Caco-2 cells were used between passage numbers 10 and 40.

Cell number was determined with a cell counter (Casy 1, Schärfe System, Reutlingen, Germany) based on conductivity measurement. Cells with more than 90% viability as assessed by the trypan blue dye exclusion test were used for analyses.

2.3. Measurement of proliferation and apoptosis

For quantitative immunoassays, cells were seeded in a 96-well microtiter plate at densities of 1×10^4 cells/well and 2×10^5 (only proliferation of PBL), respectively. After plating, Caco-2 cells were allowed to grow to 40–50% confluence (1 day). PBL and HL-60 cells were incubated with various concentrations of an individual ribonucleoside (10^{-11}–10^{-3} mol/l) for 4 h (apoptosis) or 72 h (proliferation); the respective incubation times of Caco-2 cells were 24 and 55 h. Each treatment was replicated in five wells for proliferation and three wells for apoptosis.

Proliferation changes were measured as described in [17] using a colorimetric enzyme immunoassay (Boehringer Mannheim, Germany, kit 1647229) based on incorporation of 5-bromo-2′-deoxyuridine (BrdU) during DNA synthesis within 17 h (Caco-2) or 24 h (PBL,

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HL-60). Results were read out on an ELISA spectrophotometer (Anthos HT-2) at 450 nm (reference wavelength: 620 nm).

Apoptosis was determined using a photometric immunoassay (Boehringer Mannheim, kit 1774425) which is based on the determination of histone-associated DNA fragments in the cytoplasmic fraction of cell lysates [18,19]. To distinguish apoptosis from necrosis, culture supernatant was checked simultaneously for the presence of DNA fragments. The assay was performed using monoclonal antibodies against DNA and histones, respectively, for the specific determination of mono- and oligonucleosomes according to the manufacturer's protocol, and results were read out on an ELISA spectrophotometer at 405 nm against substrate solution as a blank (reference wavelength: 492 nm).

For light microscope detection of apoptosis at single cell level the annexin V method [20] was used to detect the translocation of phosphatidylserine to the external surface of the plasma membrane with an annexin-V-biotin probe. After treatment with 10^{-3} mol/l ribonucleoside, 1×10^6 PBL or HL-60 cells were incubated in 100 μl annexin-V-biotin working solution (Boehringer Mannheim) in HEPES buffer (Sigma-Aldrich) for 10 min and washed two times. Cells were then resuspended in 0.4 ml HEPES buffer, 0.2 ml of which was transferred into a 0.75 ml cyto-container (Zyto-System, Heraeus) and centrifuged onto a slide (600 rpm, 5 min). Cells were fixed with methanol/ethanol (1:1, v/v), incubated in 100 μl streptavidin-POD working solution (Boehringer Mannheim) in HEPES buffer for 1 h, rinsed with HEPES buffer and finally incubated with DAB (diaminobenzidine) substrate solution in peroxide buffer (1+9, v/v) (Boehringer Mannheim) for 10 min. Apoptotic cells were identified by direct visualization of brown-colored membrane staining under a light microscope. The dye exclusion test using trypan blue was performed simultaneously to discriminate between apoptosis and necrosis.

Data analysis of immunoassays was performed using statistical software package StatMost (DataMost Corp., Salt Lake City, UT, USA). Comparisons of test vs. control absorbance measurements were made by Tukey's test where $P < 0.05$ was considered a significant difference of mean values. To compare the effects of different ribonucleosides, results were expressed as the increase and decrease, respectively, of cell activity in relation to control without ribonucleoside and calculated as (mean absorbance$_{test}$/mean absorbance$_{control}$).

3. Results and discussion

Attempts were made to grow cells under serum-reduced conditions and thus to avoid unwanted ribonucleoside impurities in media. Accordingly, PBL and HL-60, but not Caco-2 cells, could be adapted to a lower serum concentration without any changes in cell viability. In Caco-2 media, 0.14–0.17 μmol/l ip6ado, t6ado, guo and 2.6–3.5 μmol/l cyd as well as urd were found. However, the amount of impurities was negligible in relation to the effective concentration of such test substances which induced a cell modulating effect. In preliminary studies, cell number and incubation period with ribonucleosides were optimized to obtain a measurable modulating effect, i.e. to avoid too low or too high absorbance values. Compared with 2–4 h for induction of apoptosis in PBL and HL-60 cells, the apoptotic effect of Caco-2 cells was evident 18–24 h after culturing in the presence of an apoptosis inducing ribonucleoside.

Modulating effects of ribonucleosides were found in the range of 10^{-7}–10^{-3} mol/l. The micromolar concentration was the most effective dosage of all ribonucleosides (Table 1). Ado, m6ado, m6,2ado, ip6ado, and m2,2guo induced significant apoptosis of HL-60 cells accompanied by an inhibition of proliferation. Several ribonucleosides inhibited PBL proliferation without a simultaneous stimulation of apoptosis but a significant apoptotic and antiproliferative effect on PBL was found with m6,2ado and ip6ado. Regarding Caco-2 cell activity, m1ado, m6ado, m6,2ado, ip6ado and guo had a pronounced effect as stimulants of apoptosis as well as inhibitors of proliferation; m2guo induced significant apoptosis of Caco-2 cells but proliferation remained unchanged. It was interesting to note that the antiproliferative effect, especially in HL-60, and to some extent the enhancement of apoptosis, revealed a relationship between increasing cell reactivity and the degree of chemical modification of the adenine moiety of ado (Fig. 1, Table 1).

There is increasing evidence that apoptosis and necrosis represent the extreme ends of a wide range of possible morphological and biochemical deaths [21]. The two classical types of cell death can occur simultaneously in cell cultures exposed to the same stimulus. In this study, the mode of cell death induced by ado and several modified ribonucleosides was apoptotic. This was confirmed experimentally by the light

Table 1
Comparison of modulating effects of ribonucleosides (10^{-3} mol/l) on cell proliferation and apoptosis assayed by quantitative immunoassays based on BrdU incorporation and DNA fragmentation in cell lysates, respectively

Ribonucleoside	HL-60		PBL		Caco-2	
	Proliferation	Apoptosis	Proliferation	Apoptosis	Proliferation	Apoptosis
Ado	0.21***[a]	4.91***	0.14***	0.82	0.96	1.25
m1Ado	0.07***[b]	0.92	0.11***	0.72	0.72***	1.37**
m6Ado	0.17***[b]	1.37**	0.03***[b]	0.87	0.61***	1.34***
m6,2Ado	0.00***[c]	14.11***[a]	0.00***[c]	1.91***	0.01***[a]	5.28***
ip6Ado	0.01***[c]	21.90***[c]	0.02***[d]	2.43***	0.01***[a]	1.51
t6Ado	0.92	1.18	1.34***	0.73	0.96	1.08
Guo	0.61**	2.00	0.36***	0.83	0.89**	2.08***[c]
m1Guo	0.97	1.42	0.44***	1.06	0.88***	0.90
m2Guo	1.24*	1.58	0.71**	0.98	1.01	1.31**
m2,2Guo	0.01***	4.56***	1.41***	0.93	0.90**	0.88
m7Guo	1.26	1.71	0.15***	1.06	1.04	1.13
Ino	1.09	1.22	1.35*	0.89	1.01	1.08
m1Ino	1.16	1.22	0.71	1.09	0.98	0.97
m7Ino	0.75*	1.64	0.87	1.01	1.03	0.94
Cyd	0.59	1.70	0.73	0.84	0.81***	0.88
Urd	1.17	1.23	0.90	0.98	0.87	0.97

For details see Section 2.3. Results are expressed as the ratios test/control (mean absorbance$_{test}$/mean absorbance$_{control}$); stimulation: test/control > 1, inhibition: test/control < 1.
Mean values of measurements differ with *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.
Significant effect already measurable at ribonucleoside concentration of [a]10^{-4}, [b]10^{-5}, [c]10^{-6}, [d]10^{-7} mol/l.
[c]Accompanied by significant necrosis based on the measurement of DNA fragments in cell culture supernatant (test/control = 1.55, $P < 0.05$).

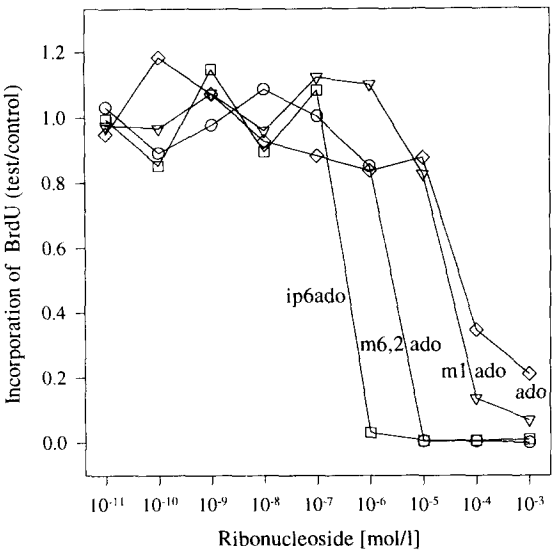

Fig. 1. Effect of modified ribonucleosides ip6ado (□), m6,2ado (○), m1ado (▽) in comparison with ado (◇) on incorporation of BrdU by human HL-60 cells. For details see Section 2.3.

microscopic proof of translocation of phosphatidylserine to the outer layer of the cell membrane (PBL, HL-60) and the lack of DNA fragments in culture supernatant (PBL, HL-60, Caco-2). Martin et al. [22] have demonstrated that the phosphatidylserine exposure is induced by a variety of apoptotic stimuli and occurs in a wide variety of cells. A significant amount of DNA fragments in culture supernatant which is indicative of the necrotic loss of cell membrane integrity and the leakage of cellular constituents into the cell environment [14] was found only with Caco-2 cells incubated with guo (Table 1). Similar to our findings in this study, Tanaka et al. [23] described the induction of apoptosis by ado (1 mmol/l) in human HL-60 cells; the adenine moiety of ado was essential for its apoptosis-inducing activity. Furthermore, they found that ado acts at the intracellular level and that the effect was not mediated by ado receptors.

We report for the first time on the in vitro modulation of apoptosis by modified ribonucleosides using human cell culture models where the hypermodified m6,2ado and ip6ado showed the highest activity. The cancer cell lines Caco-2 and HL-60 were more reactive to an apoptotic stimulation than non-malignant PBL. It is worth noting that most anticancer drugs produce apoptosis in sensitive cells [24] involving an increased apoptotic response of cancerous cells and a minimal response from normal cells. For example, micromolar concentrations of purine nucleoside analogues (2-chloro-2′-deoxyadenosine, 9-β-D-arabinosyl-2-fluoroadenine) are reported to be effective as apoptosis-inducing chemotherapeutic agents in the treatment of several types of leukemia, and it has been shown that their mechanisms of action implicate cellular signalling networks [25].

Regarding the natural presence in living systems, modified ribonucleosides are mainly found in transfer RNA and originate from cellular RNA breakdown. It is known that the pattern of these metabolic products is altered in body fluids of individuals suffering from neoplastic diseases, i.e. modified ribonucleosides serve as pathobiochemical marker molecules for cancer [9–12]. Because a vast majority of tumor promoters are potent inhibitors of apoptosis [26], it seems unlikely that cancer cells produce apoptosis-inducing ribonucleosides. It remains to be clarified if they are released by healthy cells as a reaction against malignant cells. However, this hypothesis is supported by our findings that cancer cell lines are reactive to apoptotic stimuli by modified ribonucleosides.

Dietary nucleosides are ingested mainly as nucleoproteins and are converted in the course of intestinal digestion to monomeric compounds. Ribonucleosides are in particular intrinsic components of meat and meat products [27] but they are also available as minor food components especially in human and bovine milk [2,28–31]. Food-derived inducers of apoptosis may be of significance as exogenous anti-cancerogens in the control of malignant cell proliferation where the intestinal tract could be the primary target site for a possible selective apoptotic stimuli against malignant cells.

To generalize the conclusion from the present experiments, the biospecific sensing ability of living cells provides a tool for monitoring exogenous, e.g. dietary, bioactive substances. The mechanisms through which modified ribonucleosides exert their apoptotic and potential anticancerogenic effect are unknown. Further studies are now in progress, e.g. to measure the time-dependent utilization of ribonucleosides at the extra- and intracellular level, which may give insight into the molecular mechanisms underlying the apoptosis-inducing activities of modified ribonucleosides.

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